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14. ABSTRACT

The viability of D. magna neonates (<24 h old at the start the exposure) exposed to five series of low temperatures (4-6 ºC) for periods up to 60 days was evaluated. Parthenogenetic female neonates were taken from 20 ºC to temperatures ranging from 4 to 6 ºC with continuous exposure to the low temperatures for periods of 10, 20, 30, and 60 days followed by an increase in temperature over 1-, 6-, 24-, 25- or 26-h back up 20 ºC and subsequent viability assessment via the Daphnia magna IQ Toxicity Test™. Substantial mortality (≥80%) occurred by day 20 for neonates taken from 20 to 4 ºC in 1- and 6-hour temperature decrease schemes with subsequent temperature increases of 1- and 6-h back to 20 ºC. Neonates taken from 20 ºC down to 4-6 ºC over a 24- to 26-hour period, held for 30 days at 4-6 ºC, and taken back up to 20 ºC in a 1-hour period had good survival (77-100%) and positive Daphnia IQ Toxicity Test™ responses (60 to 91%). The positive response of neonates in the Daphnia IQ Toxicity Test™ that were alive at 60 days ranged from 45 to 64%.

15. SUBJECT TERMS

Daphnia magna; neonates; cyclic temperature changes; thermal tolerance; M.S 222; ephippia; Daphnia IQ Toxicity Test™; IQ-Tox Kool Daphnia™

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Evaluation of *Daphnia magna* Neonate Viability under Low Temperature Exposure Conditions

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EXECUTIVE SUMMARY

The U.S. Army Center for Environmental Health Research (USACEHR) expressed interest in the possibility of maintaining *Daphnia magna* neonates under prolonged cold conditions (4-6 ºC). As a result, Science Applications International Corporation (SAIC) issued a Statement of Work (SOW) on July 19, 2006, which described the tasks that USACEHR wanted covered to address the maintenance of *D. magna* under cold conditions (SAIC, 2006). The University of Maryland (UMD) submitted a proposal to SAIC on July 21, 2006, to conduct the tasks described in SAIC's SOW. This report describes the result of the tasks.

USACEHR's interest in maintaining neonates under prolonged cold conditions is for possible deployment in an Environmental Sentinel Biomonitor (ESB) system. The neonates may ultimately be used in the ESB for rapid testing of potentially contaminated environmental samples via the *Daphnia magna* IQ Toxicity Test™. The thermal tolerance of *D. magna* females ranges from 3-32 ºC; however, little information is available concerning the thermal tolerance of *D. magna* neonates. Unpublished preliminary studies in our laboratory at the University of Maryland (UMD) and the recently announced Kool Daphnia™ show that *D. magna* neonates can tolerate low temperatures (4-6 ºC) for periods up to 14 days.

The primary objective of the current study was to determine the viability of *D. magna* neonates exposed to low temperatures (4-6 ºC) for periods up to 60 days. The basic experimental design for the parthenogenetic female neonates was a reduction in temperature from 20 ºC to temperatures ranging from 4 to 6 ºC with continuous exposure to the low temperatures for periods of 10, 20, 30, and 60 days followed by an increase in temperature back up 20 ºC and subsequent viability assessment via the *Daphnia magna* IQ Toxicity Test™. The following four series of temperature change were evaluated using neonates <24 hours old at the start of the exposures:

1) 20 ºC to 4 ºC over a 1-hour period followed by a return to 20 ºC over a 1-hour period at each exposure period (no food vs. food every three days was also evaluated);
2) 20 ºC to 4 ºC over a 6-hour period followed by a return to 20 ºC over a 6-hour period at each exposure period (no food vs. food every three days was also evaluated);
3) 20 ºC to 6 ºC over a 25-hour period followed by a return to 20 ºC over a 6-hour period at each exposure period (no food); and
4) 20 ºC to 4 ºC over a 26-hour period followed by a return to 20 ºC over a 1-hour period at each exposure period (no food).

A fifth series of temperature manipulations from 20 ºC down to 4 ºC followed by an increase in temperature at each exposure period back up 20 ºC in 1 hour was conducted using well fed neonates that were ~24, 48, and 96 hours old. The 24-, 48-, and 96-hour old neonates were all held at 4 ºC for periods of 10, 20, 30, and 60 days before the temperature increase back up to 20 ºC in 1 hour.

5) 20 ºC to 4 ºC over a 24-hour period followed by a return to 20 ºC over a 1-hour period at each exposure period (no food).

Substantial mortality (≥80%) occurred by day 20 for neonates taken from 20 to 4 ºC in the 1- and 6-hour temperature decrease schemes. No difference was found in survival at day 10 between the neonates in the 1st and 2nd series that were fed and not fed every three days. Thus, further tests were not conducted using the temperature manipulations in the 1st and 2nd series.

Neonates (<24 hours old at the start of the temperature manipulations) taken from 20 ºC down to 4-6 ºC over a 24 to 26-hour period and returned to 20 ºC in a 1-hour period (3rd, 4th, and 5th series) had mean survival rates ranging from 77-100% at day 30. The positive response in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive at 30 days in the three series of temperature manipulations ranged from 60 to 91%. Mean survival of the 30-day exposed neonates that were ~48- and 96-hours old was 50 and 37%, respectively, while the positive response in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive was 73% in both the ~48- and 96-hour old neonates.

Mean survival at 60 days was 73% in the neonates (<24 hours old at the start of the temperature manipulations) taken from 20 to 6 ºC in 26 hours (3rd series) and returned to 20 ºC in one hour. In contrast, the mean survival of the 24-hour old neonates at 60 days taken from 20 to 4 ºC in 24 hours was only 33 and 37%, respectively, in the 4th and 5th series. The positive response of the 24-hour old neonates in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive at 60 days ranged from 45 to 64% in the three series of temperature manipulations. Survival was poor (13%) in the ~48- and 96-hour old neonates in the 5th series at 60 days; while the positive *Daphnia* IQ Toxicity Test™ responses of the neonates still alive in the ~48- and 96-hour exposures were 50% in both exposures.

In conclusion, the low temperature manipulation studies show that neonates (<24 hours old at the start of the temperature manipulations) can be taken from 20 ºC down to 4-6 ºC over a 24- to 26-hour period, held for 30 days at 4-6 ºC, and taken back up to 20 ºC in a 1-hour period with good survival (77-100%) and positive *Daphnia* IQ Toxicity Test™ responses (60 to 91%). With the exception of survival (73%) in neonates taken down to 6 ºC (3rd series), survival was poor (33-37%) in the 4th and 5th series at 60 days. The positive response of the 24-hour old neonates in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive at 60 days ranged from 45 to 64% in the three series of temperature manipulations.

In addition to the temperature manipulation studies, experiments were also conducted with neonates exposed to a number of tricaine methanesulfonate (M.S. 222) concentrations to determine if the neonates could be anesthetized prior to the temperature manipulations and thus better survive the temperature changes. Neonates were exposed at 20 ºC to a series of M.S. 222 concentrations ranging from 1-800 mg/L for 24 or 48 hours and observed for recovery in clean water for periods of 24 or 48 hours. Exposure to M.S. 222 concentrations of 1, 10, and 20 mg/L for 48 hours did not anesthetize any neonates. Concentrations between 50 and 150 mg/L M.S. 222 anesthetized increasing numbers of the neonates as a function of the dose (0% anesthetized at 50 mg/L up to 75% anesthetized at 150 mg/L) in 24 hours; 100% of the neonates were anesthetized by 48 hours at all of the concentrations between 50 and 150 mg/L. A small percentage (<25%) survived at 24 hours in fresh water; however, none of the neonates recovered (all neonates died) after 48 hours in clean water in the 50-150 mg/L exposures. M.S. 222 concentrations of 200, 400, and 800 mg/L anesthetized all neonates in one hour. None of the

neonates recovered in 24 hours in clean water. A few neonates at 200 mg/L exposed for 1 and 2 hours exhibited a "twitching" of the antennae but no other movement or heart beat. The experiments with M.S. 222 showed that neonates can be anesthetized; however, the animals that are anesthetized do not readily recovery from the anesthesia when placed in clean water. Thus, there is no advantage in anesthetizing the neonates prior to lowering the temperatures from 20 ºC to 4-6 ºC.

A small effort was also made to produce *D. magna* ephippia for use in the production of neonates. An outdoor mesocosm (33 gallon barrel with ~25 gallons of pond water), which used overcrowding of females, reduction in temperature, and the possible presence of fish kairomones as stimuli for ephippia production, produced a limited number of males and ephippia (several dozen) when the ambient temperatures began to fall below 12-15 ºC. Several dozen ephippia were also produced in approximately two weeks in small (1- and 4-L) laboratory mesocosms (overcrowded with females) and maintained under a 24-hour continuous light photoperiod produced. No attempt was made to determine hatching success of the ephippia.

1.0 INTRODUCTION

The U.S. Army Center for Environmental Health Research (USACEHR) expressed interest in the possibility of maintaining *Daphnia magna* neonates under prolonged cold conditions (4-6 ºC). As a result, Science Applications International Corporation (SAIC) issued a Statement of Work (SOW) on July 19, 2006, which described the tasks that USACEHR wanted covered to address the maintenance of *D. magna* under cold conditions (SAIC, 2006). The University of Maryland (UMD) submitted a proposal to SAIC on July 21, 2006, to conduct the tasks described in SAIC's SOW. This report describes the result of the tasks.

USACEHR's interest in maintaining neonates under prolonged cold conditions is for possible deployment in an Environmental Sentinel Biomonitor (ESB) system. The neonates may ultimately be used in the ESB for rapid testing of potentially contaminated environmental samples via the *Daphnia magna* IQ Toxicity Test™. The thermal tolerance of *D. magna* females ranges from 3-32 ºC; however, little information is available concerning the thermal tolerance of *D. magna* neonates. Kingwood Diagnostics recently announced (November 2, 2006) that they have developed an IQ-Tox Kool Daphnia™ test kit (Kool Daphnia™) which utilizes daphnids that have a shelf life of 14 days when the organisms are held between 4 and 6 ºC and fed a supply of algal food once each week (Kingwood Diagnostics, 2006a). Unpublished preliminary studies in our laboratory at the UMD also show that neonates can tolerate low temperatures (4-6 ºC) for periods up to 14 days without the addition of algae. USACEHR's minimal shelf life requirement for the use of *D. magna* in the ESB is 30 days at 4-6 ºC with no feeding; 60 days would be preferable to 30 days (Shedd, 2006).

The thermal tolerance of *D. magna* females ranges from 3-32 ºC; however, the adults do not appear to over winter (Mitchell et al., 2004). Little information is available concerning the thermal tolerance of *D. magna* neonates. Unpublished preliminary studies in our laboratory at the University of Maryland and the recently announced Kool Daphnia™ show that *D. magna* neonates can tolerate low temperatures (4-6 ºC) for periods up to 14 days. A Google Scholar™ literature search revealed that no systematic data are available concerning the rates of temperature reduction at which adults and neonates may acclimatize or acclimate to low temperatures (Google, 2006). Ancillary data are available for thermal stress in ponds and lakes.

Limited studies indicate that some species of cladoceran females may over winter and produce neonates in the spring. Most of the literature, however, shows that ephippia formation occurs and may be attributable to a number of factors, including overcrowding, reduction in food supply, reduction in temperature, change in photoperiod, increased predation risk, kairomone release by fish, and in some cases, a combination of the known factors (Carvalho and Hughes, 1983; Pijanowska and Stolpe, 1996; Slusarczyk, 1995, 1999, 2001; and others). When "conditions" become unfavorable, normally parthenogenetic females go into sexual reproduction and start producing haploid eggs and males. "Resting" eggs (usually two eggs in *D. magna*) are then produced and held in the ephippia.

The primary objective of the current study was to determine the viability of *D. magna* neonates exposed to low temperatures (4-6 ºC) for periods up to 60 days. Several sets of experiments were performed to meet the objective of the study. They included 1) a determination of the

viability of neonates produced from healthy parthenogenetic females acclimated to 20 ºC and exposed to a reduction in temperature from 20 ºC down to 4-6 ºC at several rates of temperature change; 2) how long the neonates survived at the low temperatures; and 3) how viable the neonates were upon return to 20 ºC at two rates of temperature change. The *Daphnia* IQ Toxicity Test™ (Kingwood Diagnostics, 2006b) was used to assess viability. A set of experiments was also conducted with neonates exposed to an anesthetic agent to determine if neonates could be anesthetized prior to the temperature manipulations and thus better survive the temperature changes. In addition, a small effort was made to produce *D. magna* ephippia in the laboratory. Ephippia can be stored at low temperatures for extended periods of time (years) and later used be used for the production of neonates (Pennak, 1989).

2.0 MATERIALS AND METHODS

2.1 Source of Neonates

Neonates were obtained from UMD in-house cultures containing healthy well fed parthenogenetic females that were cultured via the method given in USEPA (2002). Briefly, the adults were cultured at room temperature (~20 ºC) under a 16 h light:8 h dark photoperiod (50-100 ft-c) in 4-L glass beakers containing 3 L of non-chlorinated well water (pH 7.2-7.8; hardness 70-80 mg/L as $CaCO_3$). The water in each culture vessel was replaced three times each week with fresh water. The cultures were fed a mixture of 4.5 mL YCT (yeast, Cerophyll®, and trout chow) and 2 mL of the green alga, S*elenastrum capricornutum* in log growth phase at each renewal. All cultures were thinned whenever the population exceeded 200 individuals per stock vessel to prevent over crowding which may cause a population crash and/or production of ephippia. A minimum of five cultures were maintained throughout the study.

2.2 Quality Assurance

Quality assurance was assessed by conducting four periodic 21-d life-cycle tests over the course of the studies described below following the procedures given in ASTM (2004). Each of the four life-cycle tests consisted of two simultaneous tests of 12 replicates per test (one neonate per beaker). The life-cycle tests were initiated with neonates <24 hours taken from a brood stock that had been cultured for at least two generations under the conditions described in Section 2.1. All tests were conducted in a constant temperature room (20 ºC) under a 16 h light:8 h dark photoperiod (50-100 ft-c). Each neonate was placed in a 50 mL beaker containing 40 mL well water. The water in each replicate was renewed every other day with fresh water. Each replicate was fed a mixture of YCT and S. *capricornutum* in log growth phase daily. Dissolved oxygen and pH were randomly measured daily in one beaker in each test. Hardness, alkalinity, and conductivity were randomly measured at approximately weekly intervals. Temperature was recorded continuously throughout the test.

2.3 Viability of Parthenogenetic Female Neonates Exposed To Low Temperatures

The basic experimental design for the parthenogenetic female neonates was a reduction in room temperature from 20 ºC to temperatures ranging from 4 to 6 ºC with continuous exposure to the low temperatures for periods of 10, 20, 30, and 60 days followed by an increase in temperature

back up 20 ºC. The following four series of temperature change were evaluated using neonates <24 hours old at the start of the exposures:

1) 20 ºC to 4 ºC over a 1-hour period followed by a return to 20 ºC over a 1-hour period at each exposure period (no food vs. food every three days was also evaluated);
2) 20 ºC to 4 ºC over a 6-hour period followed by a return to 20 ºC over a 6-hour period at each exposure period (no food vs. food every three days was also evaluated);
3) 20 ºC to 6 ºC over a 25-hour period followed by a return to 20 ºC over a 6-hour period at each exposure period (no food); and
4) 20 ºC to 4 ºC over a 26-hour period followed by a return to 20 ºC over a 1-hour period at each exposure period (no food).

A fifth series of temperature manipulations from 20 ºC down to 4 ºC followed by an increase in temperature at each exposure period back up 20 ºC in 1 hour was conducted using well fed neonates that were ~24, 48, and 96 hours old. The 24-, 48-, and 96-hour old neonates were all held at 4 ºC for periods of 10, 20, 30, and 60 days before the temperature increase back up to 20 ºC in 1 hour.

5) 20 ºC to 4 ºC over a 24-hour period followed by a return to 20 ºC over a 1-hour period at each exposure period (no food).

The temperature decreases in the 1st and 2nd series were essentially linear over the 1- and 6-hour reduction periods. The temperature reductions over time in the 3rd, 4th, and 5th series are shown in Figure 1. A linear line has been superimposed over the actual data plots to show the variations from linearity.

All neonates were held in the dark at the low temperatures during all exposure periods. All neonates were offered food as described in Section 2.1 prior to the start of the temperature manipulations. No food versus food every three days during the low temperature exposures were evaluated in the 1st and 2nd temperature manipulations shown above. In the food versus no food experiments, 1 mL of YCT plus algae (50:50 mix) was added to each replicate every three days. No attempt was made to remove the food after it was added.

The neonates were not fed while at the low temperatures during the 3rd, 4th, and 5th series of temperature manipulations. Survival upon return to 20 ºC was scored as those neonates that were mobile and/or had an active heart beat. Viability upon return to 20 ºC was determined by the *Daphnia* IQ Toxicity Test™. The viability of the experimental neonates brought back up to 20 ºC was compared to reference neonates (24-48 hours old) cultured at 20 ºC. That is, the temperature-manipulated neonate's fluorescence was scored against the fluorescence of constant temperature reference neonates.

Three replicates of 15 neonates per replicate (each replicate of 15 neonates was placed in a 150 mL beaker containing 120 mL of water) were exposed to the 1st and 2nd temperature manipulations for periods of 10, 20, and 30 days. No animals survived at 30 days; thus, the 60-day period was terminated. Six neonates were randomly selected from each of the three replicates for the *Daphnia* IQ Toxicity Test™ during the first two sets of temperature

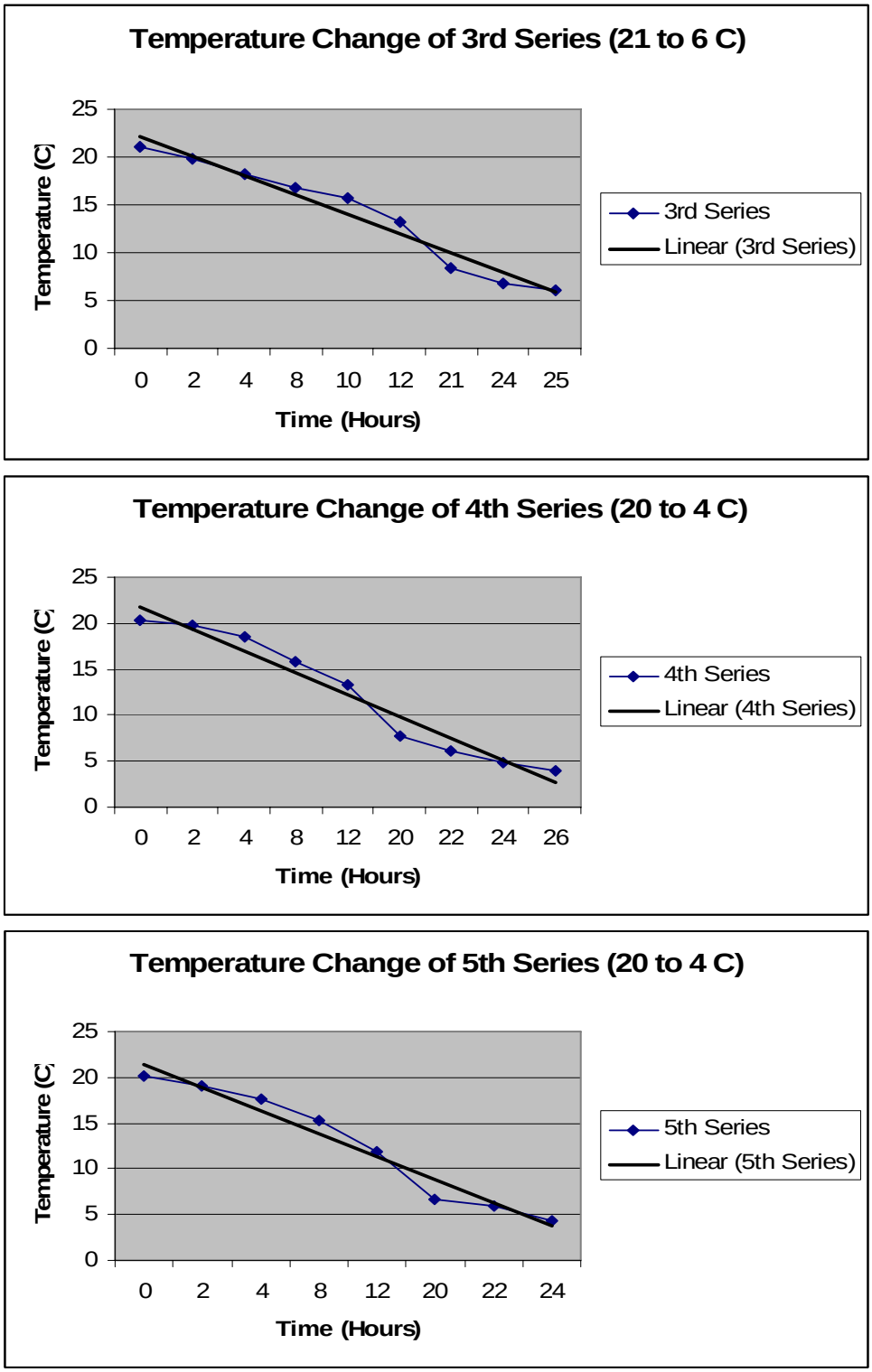

Figure 1. Plots of the Temperature Decreases in the 3rd, 4th, and 5th Series of Exposures

manipulations. The Kingwood Diagnostics Instruction Manual for the *Daphnia* IQ Toxicity Test™ states that neonates should not be fed six to 24 hours prior to testing because a starvation period produces more consistent organism response and faster scoring (Kingwood Diagnostics, 2006b). Since the neonates had been held at each exposure period with no food in most cases, the neonates were evaluated immediately (within one hour) upon return to 20 ºC.

Three replicates of 10 neonates per replicate (each replicate of 10 neonates was placed in a 150 mL beaker containing 120 mL of water) were exposed to the 3rd, 4th, and 5th series of temperature manipulations for periods of 10, 20, 30, and 60 days. In contrast to the 1st and 2nd tests in which six organisms were randomly selected for the *Daphnia* IQ Toxicity Test™, all living neonates in each replicate at each exposure period were tested via the *Daphnia* IQ Toxicity Test™ in the 3rd, 4th, and 5th series of temperature manipulations. Viability was evaluated immediately (within one hour) upon return to 20 ºC. Temperature was measured at various intervals during the temperature decreases and continuously at the low exposure temperatures. pH and dissolved oxygen were measured at the beginning (day 0) of the five temperature manipulations, at the end of each exposure period at 4-6 ºC, and again at 20 ºC. Conductivity, alkalinity, and hardness were measured only at the start (day 0) of the five temperature manipulations.

2.4 Viability of Parthenogenetic Female Neonates Exposed to an Anesthetic (M.S. 222)

Neonates produced from parthenogenetic females (<24 hours old at start of the exposures) were exposed to a number of tricaine methanesulfonate (M.S. 222) concentrations to determine if the neonates could be anesthetized prior to the temperature manipulations and thus better survive the temperature changes. Quinaldine was rejected for consideration because a solvent (e.g., acetone or ethanol) is recommended since the compound is only slightly soluble in water. Two sets of exposures were used. The first set consisted of exposures for periods of 1, 2, 4, 24, and 48 hours. If the animals were still alive after being exposed to the anesthetic, they were subsequently placed in clean water containing no anesthetic and observed for recovery at 24 and 48 hours. The second set consisted of exposures for periods of 1, 2, 4, and 24 hours and a 24-hour recovery period in clean water containing no anesthetic.

Two replicates of 10 neonates per replicate (each replicate of 10 neonates was placed in a 150 mL beaker containing 120 mL of water) were used for all exposures. The animals were not fed during the exposure periods or during the periods in clean water. All tests were conducted at 20 ºC under a 16h light:8h dark photoperiod in UMD well water. The endpoint for the exposures was survival (active heart beat, motility, and/or "twitching" antennae). Viability was not evaluated in the first set of exposures via the *Daphnia* IQ Toxicity Test™ after the exposure and recovery periods in the cases where organisms were still living and <6 neonates were alive at the end of the recovery periods since six neonates are the minimum recommended for the *Daphnia* IQ Toxicity Test™. Viability was evaluated with <6 neonates in the second set of exposures. Control replicates of 10 neonates/replicate (<24 hours old at start of the exposures) were held at 20 ºC under a 16h light:8h dark photoperiod in clean water during each set of exposures. Viability of the control neonates was evaluated via the *Daphnia* IQ Toxicity Test™ at the end of the exposure and recovery periods for the experimental organisms. That is, the control neonates were the same age that the experimental animals would have been if they had survived the M.S.

222 exposures. Routine water chemistry (pH, dissolved oxygen, conductivity, alkalinity, and hardness) was taken at the beginning of the exposure and recovery periods.

2.5 Production of Ephippia

The following efforts were made to produce ephippia:

1) An outdoor *D. magna* mesocosm was started in mid-August in a 33 gallon drum containing ~25 gallons of UMD non-chlorinated deep well water. The mesocosm was overcrowded with several hundred parthenogenetic females/L taken from UMD cultures. Filtered pond water was used as a source of food, which should also have contained bluegill (*Lepomis macrochirus*) kairomones that are thought to induce sexual reproduction. Approximately 75% of the 25-gallon volume was exchanged two times each week. The mesocosm was kept outside at ambient temperatures under ~10 hours sunlight. The mesocosm was checked once a week for adults and ephippia by siphoning up and down the water column and passing the water over a series of screens. Approximately 200 adults were examined each week to estimate the number of males present. Ephippia were collected on a 150 µm screen;
2) Several 1- and 4-L laboratory mesocosms containing well water at room temperature under a 16h light:8h dark photoperiod (50-100 ft-c at the surface of the water) were established in the laboratory. The mesocosms were overcrowded with several hundred parthenogenetic females/L taken from UMD cultures as the initiator for ephippia production. The water in each culture vessel was replaced three times each week with fresh water. The cultures were fed a mixture of 4.5 mL YCT and 2 mL of *S. capricornutum* in log growth phase at each renewal; and
3) Several 1- and 4-L laboratory mesocosms containing well water at room temperature were held under a continuous 24-hour photoperiod (50-100 ft-c at the surface of the water). The same test conditions described above were also used in the continuous 24-hour light photoperiod mesocosms.

3.0 RESULTS AND DISCUSSION

3.1 Quality Assurance

The UMD in-house *D. magna* in the four series of 21-d life-cycle tests met the control test acceptability criteria specified in Section 14 of ASTM (2004). This indicates that the UMD cultures were healthy and that the neonates used in the various experiments should give repeatable results. All 12 replicates in all tests met the criterion of a minimum of 60 neonates in 21 days. A summary of the mean number of broods and mean total number of young produced in tests 1 and 2 of the four life-cycle test is given in Table 1. The actual number of broods and neonates produced in each of the 12 replicates in tests 1 and 2 as well as the water quality of each of the four life-cycle tests are given in Attachments I-IV.

3.2 Viability of Parthenogenetic Female Neonates Exposed To Low Temperatures

A summary of neonate survival and *Daphnia* IQ Toxicity Test™ results for each of the five

Table 1. Mean Number of Broods and Young Produced in the Four 21-Day Life-Cycle Tests (Mean of 12 Replicates)

Test	Mean No. of Broods	Mean No. of Young Produced
	1st Series (July 28, 2006 - August 18, 2006)	
1	6.8	146.5
2	6.9	136.4
	2nd Series (September 19, 2006 - October 21, 2006)	
1	6.7	87.6
2	6.7	90.2
	3rd Series (October 16, 2006 - November 6, 2006)	
1	6.1	83.5
2	6.3	82.2
	4th Series (November 6, 2006 - November 27, 2006)	
1	6.1	103.4
2	6.3	106.3

series of temperature manipulations is given in Tables 2-6. The water quality data during the five series of temperature manipulations are given in Attachment V. Survival was substantially less in the neonates exposed in the first two series of temperature reductions from 20 to 4 ºC over the 1- and 6-h periods (Tables 2 and 3) relative to the organisms exposed to the temperature reductions in the 3rd, 4th, and 5th series over 24 to 26-hours (Tables 4-6). The mean survival after 10 days at 4 ºC in the 1- and 6-hour reductions in the replicates that did not receive food was 71 and 82%, respectively. Mean survival after 10 days at 4 ºC in the 1- and 6-hour reductions that received food was 73 and 87%, respectively. No difference ($p<0.05$; two-tailed t-test; WEST Gulley, 1996) occurred at 10 days between the organisms receiving no food versus food in either the 1- or 6-hour treatment. Of the experimental organisms that survived at 10 days, 1-2 in each replicate were alive but not active (heart beat; little locomotion). With the exception of one replicate in the 1-hour treatment, all six randomly selected neonates in each replicate of the 1- and 6-hour treatments as well as the reference neonates gave a positive response in the *Daphnia* IQ Toxicity Test™ at 10 days.

Mean survival at 20 days of exposure at 4 ºC was 0 and 18% for the organisms that did not receive food in the 1- and 6-hour treatments, respectively, while the mean for the animals that received food was 2 and 20% in the 1- and 6-hour treatments (Tables 2 and 3). Approximately 55% of the neonates that survived at 20 days gave a positive response in the *Daphnia* IQ Toxicity Test™ (Tables 1 and 2). No neonates survived in either treatment at 30 days. All randomly selected reference neonates gave a positive response in the *Daphnia* IQ Toxicity Test™ at 10, 20, and 30 days.

Table 2. Summary of Neonate Survival and *Daphnia* IQ Toxicity Test™ Results in the 1st Series of Temperature Manipulations from 20 to 4 ºC in 1 Hour (Returned to 20 ºC in 1 Hour)

Treatment	Rep	Survival	Percent Survival	No. Alive, Inactive	IQ Test (+)
	Day 10 of Cold Exposure				
No Food	A	11/15	73.3	2	6/6
	B	10/15	66.7	2	5/6
	C	11/15	73.3	2	6/6
Food	A	12/15	80.0	2	6/6
	B	10/15	66.7	2	5/6
	C	11/15	73.3	1	6/6
Reference Neonates[a]	A	N/A	N/A	N/A	6/6
	B	N/A	N/A	N/A	6/6
	C	N/A	N/A	N/A	6/6
	Day 20 of Cold Exposure				
No Food	A	0/15	0	-	-
	B	0/15	0	-	-
	C	0/15	0	-	-
Food	A	1/15	6.7	0	1/1
	B	0/15	0	-	-
	C	0/15	0	-	-
Reference Neonates[a]	A	N/A	N/A	N/A	6/6
	B	N/A	N/A	N/A	6/6
	C	N/A	N/A	N/A	6/6
	Day 30 of Cold Exposure				
No Food	A	0/15	0	-	-
	B	0/15	0	-	-
	C	0/15	0	-	-
Food	A	0/15	0	-	-
	B	0/15	0	-	-
	C	0/15	0	-	-
Reference Neonates[a]	A	N/A	N/A	N/A	6/6
	B	N/A	N/A	N/A	6/6
	C	N/A	N/A	N/A	6/6

[a] All reference neonates were 24-48 hours old when tested.

Table 3. Summary of Neonate Survival and *Daphnia* IQ Toxicity Test™ Results in the 2nd Series of Temperature Manipulations from 20 to 4 ºC in 6 Hours (Returned to 20 ºC in 6 Hours)

Treatment	Rep	Survival	Percent Survival	No. Alive, Inactive	IQ Test (+)
	Day 10 of Cold Exposure				
No Food	A	12/15	80.0	1	6/6
	B	12/15	80.0	2	6/6
	C	13/15	86.7	2	6/6
Food	A	12/15	80.0	1	6/6
	B	13/15	86.7	1	6/6
	C	13/15	86.7	2	5/6
Reference Neonates[a]	A	N/A	N/A	N/A	6/6
	B	N/A	N/A	N/A	6/6
	C	N/A	N/A	N/A	6/6
	Day 20 of Cold Exposure				
No Food	A	2/15	13.3	0	1/2
	B	4/15	26.7	0	2/4
	C	2/15	13.3	0	1/2
Food	A	2/15	13.3	0	1/2
	B	5/15	33.3	1	2/4
	C	2/15	13.3	1	1/1
Reference Neonates[a]	A	N/A	N/A	N/A	6/6
	B	N/A	N/A	N/A	6/6
	C	N/A	N/A	N/A	6/6
	Day 30 of Cold Exposure				
No Food	A	0/15	0	-	-
	B	0/15	0	-	-
	C	0/15	0	-	-
Food	A	0/15	0	-	-
	B	0/15	0	-	-
	C	0/15	0	-	-
Reference Neonates[a]	A	N/A	N/A	N/A	6/6
	B	N/A	N/A	N/A	6/6
	C	N/A	N/A	N/A	6/6

[a] All reference neonates were 24-48 hours old when tested.

Table 4. Summary of Neonate Survival and *Daphnia* IQ Toxicity Test™ Results in the 3rd Series of Temperature Manipulations from 20 to 6 ºC in 25 Hours (Returned to 20 ºC in 6 Hours)

Treatment	Rep	Survival	Percent Survival	No. Alive, Inactive	IQ Test (+)
	Day 10 of Cold Exposure				
Experimental	A	10/10	100	0	10/10
	B	10/10	100	0	9/10
	C	10/10	100	0	10/10
Reference Neonates[a]	A	N/A	N/A	N/A	10/10
	Day 20 of Cold Exposure				
Experimental	A	10/10	100	0	8/10
	B	10/10	100	0	10/10
	C	10/10	100	0	9/10
Reference Neonates[a]	A	N/A	N/A	N/A	10/10
	Day 30 of Cold Exposure				
Experimental	A	10/10	100	0	6/10
	B	10/10	100	0	7/10
	C	10/10	100	0	5/10
Reference Neonates[a]	A	N/A	N/A	N/A	10/10
	Day 60 of Cold Exposure				
Experimental	A	8/10	80	0	3/8
	B	7/10	70	0	4/7
	C	7/10	70	0	3/7
Reference Neonates[a]	A	N/A	N/A	N/A	10/10

[a] All reference neonates were 24-48 hours old when tested.

Table 5. Summary of Neonate Survival and *Daphnia* IQ Toxicity Test™ Results in the 4th Series of Temperature Manipulations from 20 to 4 ºC in 26 Hours (Returned to 20 ºC in 1 Hour)

Treatment	Rep	Survival	Percent Survival	No. Alive, Inactive	IQ Test (+)
	Day 10 of Cold Exposure				
Experimental	A	10/10	100	0	10/10
	B	10/10	100	0	9/10
	C	9/10	90	0	8/9
Reference Neonates[a]	A	N/A	N/A	N/A	10/10
	Day 20 of Cold Exposure				
Experimental	A	9/10	90	0	8/9
	B	8/10	80	0	8/8
	C	9/10	90	0	8/9
Reference Neonates[a]	A	N/A	N/A	N/A	10/10
	Day 30 of Cold Exposure				
Experimental	A	8/10	80	0	7/8
	B	8/10	80	0	8/8
	C	7/10	70	0	6/7
Reference Neonates[a]	A	N/A	N/A	N/A	10/10
	Day 60 of Cold Exposure				
Experimental	A	4/10	40	0	2/4
	B	2/10	20	0	1/2
	C	4/10	40	0	3/4
Reference Neonates[a]	A	N/A	N/A	N/A	10/10

[a] All reference neonates were 24-48 hours old when tested.

Table 6. Summary of Neonate Survival and *Daphnia* IQ Toxicity Test™ Results in the 5th Series of Temperature Manipulations from 20 to 4 ºC in 24 Hours (Returned to 20 ºC in 1 Hour)

Treatment	Rep	~24 Hours Old			~48 Hours Old			~96 Hours Old		
		Survival	No. Alive, Inactive	IQ Test (+)	Survival	No. Alive, Inactive	IQ Test (+)	Survival	No. Alive, Inactive	IQ Test (+)
Day 10 of Cold Exposure										
Experimental	A	10/10	0	10/10	10/10	0	10/10	10/10	0	10/10
	B	10/10	0	9/10	10/10	0	10/10	10/10	0	10/10
	C	9/10	0	9/10	10/10	0	10/10	10/10	0	10/10
R. neonates[a]	A	N/A	N/A	10/10	N/A	N/A	10/10	N/A	N/A	10/10
Day 20 of Cold Exposure										
Experimental	A	9/10	0	8/9	8/10	0	7/8	8/10	0	6/8
	B	9/10	0	9/9	7/10	0	6/7	7/10	0	6/7
	C	9/10	0	8/9	8/10	0	6/8	6/10	0	5/6
R. neonates[a]	A	N/A	N/A	10/10	N/A	N/A	10/10	N/A	N/A	10/10
Day 30 of Cold Exposure										
Experimental	A	8/10	0	7/8	5/10	0	4/5	4/10	0	3/4
	B	7/10	0	6/7	6/10	0	4/6	3/10	0	2/3
	C	8/10	0	7/8	4/10	0	3/4	4/10	0	3/4
R. neonates[a]	A	N/A	N/A	10/10	N/A	N/A	10/10	N/A	N/A	10/10
Day 60 of Cold Exposure										
Experimental	A	4/10	0	3/4	1/10	0	1/1	3/10	0	2/3
	B	3/10	0	2/3	1/10	0	0/1	0/10	0	-
	C	4/10	0	2/4	2/10	0	1/2	1/10	0	0/1
R. neonates[a]	A	N/A	N/A	10/10	N/A	N/A	10/10	N/A	N/A	10/10

[a] R. neonates = reference neonates which were 24-48 hours old when tested.

All neonates in the 3rd series survived when taken from 20 to 6 ºC over a 25-hour period and back up to 20 ºC in a 6-hour period at 10, 20, and 30 days; 73% survived at day 60 (Table 4). All animals were active when brought back up to 20 ºC. At days 20, 30, and 60, a few neonates were larger than the majority of neonates which indicates that they probably molted over the course of the exposures. Unfortunately, at the time the observations were made, we did not look for discarded carapaces that would have confirmed molting. In contrast to the 1st and 2nd series of temperature manipulations in which six neonates were randomly selected and tested for viability via the *Daphnia* IQ Toxicity Test™, all living neonates in each replicate were tested for viability in the current temperature manipulation. The positive response of three replicates in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive at 10, 20, 30, and 60 days was 97, 90, 60, and 45% respectively. The neonates that molted during the low temperature exposure gave a positive response in contrast to the smaller neonates which were alive but did not respond. All of the reference neonates gave a positive response in the *Daphnia* IQ Toxicity Test™ at 10, 20, 30, and 60 days.

Mean neonate survival in the 4th series when temperatures were taken from 20 to 4 ºC in 26 hours and returned to 20 ºC in 1 hour at 10, 20, 30, and 60 days was 97, 87, 77, and 33 %, respectively (Table 5). The positive response of the three replicates in the *Daphnia* IQ Toxicity Test™ of neonates that were alive at 10, 20, 30, and 60 days was 93, 92, 91, and 60% respectively. All of the reference neonates gave a positive response in the *Daphnia* IQ Toxicity Test™ at 10, 20, 30, and 60 days. As was the case in the 3rd series, all living neonates in each replicate were tested for viability in the current series of temperature manipulations. In contrast to the 3rd series where some of the neonates were larger by day 20 and thus indicated that some of the neonates molted, the neonates in the 4th series were all of uniform size at days 20, 30, and 60. Unfortunately, we did not look for discarded carapaces that would have confirmed molting.

Neonate survival in the 5th series when temperatures were taken from 20 to 4 ºC in 24 hours and returned to 20 ºC in 1 hour and well fed neonates ~24, 48, and 96 hours old were evaluated is summarized in Table 6. Mean survival (three replicates) of the ~24-hour old neonates at 10, 20, 30, and 60 days of exposure was 97, 90, 77, and 37 %, respectively. The positive response in the *Daphnia* IQ Toxicity Test™ for the ~24 hour old neonates that were alive at 10, 20, 30, and 60 days was 93, 93, 87, and 64 %, respectively (Table 6). The mean survival for the ~48 hour old neonates at days 10, 20, 30, and 60 was 100, 77, 50, and 13 %, respectively. The positive response in the *Daphnia* IQ Toxicity Test™ for the ~48-hour old neonates at 10, 20, 30, and 60 days was 100, 83, 73, 50 %, respectively. The mean survival for the ~96 hour old neonates at days 10, 20, 30, and 60 was 100, 70, 37, and 13 %, respectively. The positive response in the *Daphnia* IQ Toxicity Test™ for the ~96-hour old neonates at 10, 20, 30, and 60 days was 100, 81, 73, and 50 %, respectively. All of the reference neonates gave a positive response in the *Daphnia* IQ Toxicity Test™ at 10, 20, 30, and 60 days.

Molting was observed in some of the neonates in this series of temperature changes. The ~24-hour old neonates did not appear to molt at day 10 and 20; however, all of the living neonates molted by day 30 (carapaces were observed). The ~48-and 96-hour old neonates did not molt by day 10; however, all organisms molted by day 20 (carapaces were observed).

In summary, substantial mortality (≥80%) occurred by day 20 for neonates (<24 hours old at start of exposure) taken from 20 to 4 ºC in the 1- and 6-hour temperature decrease schemes. Thus, further tests were not conducted using the temperature manipulations in the 1st and 2nd series. A number of the neonates in the 1- and 6-hour series were observed to be alive (heart beat) at day 10 and 20 but were immobile (inactive). This phenomenon was not observed in the 3rd, 4th, and 5th series of temperature manipulations.

In the 3rd series of temperature manipulations where the neonates were taken from 20 to 6 ºC in 25 hours and returned to 20 ºC in 6 hours and the 4th series where the neonates were taken from 20 to 4 ºC in 26 hours and returned to 20 ºC in 1 hour, all animals survived at 20 days in both series. At 30 days 100% survival occurred in the 3rd series and 77% survival occurred in the 4th series. The positive response in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive at day 20 day was 90 and 92%, respectively, for the 3rd and 4th series of tests and at day 30 it was 60 and 91% in the 3rd and 4th series, respectively. Seventy-three percent of the neonates survived the 60 days of exposure at 6 ºC in the 3rd series; the positive response in the *Daphnia* IQ Toxicity Test™ was 45%. Thirty-three percent of the animals survived the 60-day exposure at 4 ºC in the 4th series; the positive response in the *Daphnia* IQ Toxicity Test™ of the neonates that were still alive was 60%.

In the 5th series where the temperature was taken from 20 to 4 ºC in 24 hours and returned to 20 ºC in 1 hour, 97-100% of the ~24-, 48-, and 96-hour old neonates survived at day 10. Survival ranging from 97-100% was also found in the 3rd and 4th series at day 10. The positive response in the *Daphnia* IQ Toxicity Test™ at day 10 in the 5th series ranged from 93-100%; similar results were found in the 3rd and 4th series at day 10. At day 20, 100% survival occurred for neonates in the 3rd series, 87% in the 4th series, and 90% in the 5th series for the ~24-hour old neonates. Survival was less for the ~48-hour old (77%) and ~96-hour old (70%) neonates in the 5th series at day 20. The positive response in the *Daphnia* IQ Toxicity Test™ for the ~48- and 96-hour old neonates that were alive was 83 and 81% at day 20 compared to 93% in the ~24-hour old neonates.

The mean survival in the 5th series at day 30 in the ~24-, 48-, and 96-hour old neonates was 77, 50, and 37%, respectively. The positive response in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive at day 30 was 87, 73, and 73%, respectively, in the ~24-, 48-, and 96-hour old neonates. The mean survival in the 5th series at day 60 in the ~24-, 48-, and 96-hour old neonates was 37, 13, and 13%, respectively. The positive response in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive at day 60 was 64, 50, and 50% in the ~24-, 48-, and 96-hour old neonates.

In summary, the neonates (<24 hours old at the start of the temperature manipulations) taken from 20 ºC down to 4-6 ºC over a 24 to 26-hour period and returned to 20 ºC in a 1-hour period (3rd, 4th, and 5th series) had mean survival rates ranging from 77-100% at day 30. The positive response in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive at 30 days in the three series of temperature manipulations ranged from 60 to 91%. Mean survival of the 30-day exposed neonates that were ~48- and 96-hours old was 50 and 37%, respectively, while the positive response in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive was 73% in both the ~48- and 96-hour old neonates.

Mean survival at 60 days was 73% in the neonates (<24 hours old at the start of the temperature manipulations) taken from 20 to 6 ºC in 26 hours (3rd series) and returned to 20 ºC in one hour. In contrast, the mean survival of the 24-hour old neonates at 60 days taken from 20 to 4 ºC in 24 hours was only 33 and 37%, respectively, in the 4th and 5th series. The positive response of the 24-hour old neonates in the *Daphnia* IQ Toxicity Test™ of the neonates that were alive at 60 days ranged from 45 to 64% in the three series of temperature manipulations. Survival was poor (13%) in the ~48- and 96-hour old neonates in the 5th series at 60 days; while the positive *Daphnia* IQ Toxicity Test™ responses of the neonates still alive in the ~48- and 96-hour exposures were 50% in both exposures.

3.3 Viability of Parthenogenetic Female Neonates Exposed to an Anesthetic (M.S. 222)

A summary of the neonates that were anesthetized with M.S 222 during the first set of exposures (1, 2, 4, 24, and 48 hours) and observed for recovery at 24 and 48 hours in clean water is given in Table 7. The second set of exposures for periods of 1, 2, 4, and 24 hours followed by a 24-hour recovery period is summarized in Table 8. The water quality for both sets of exposures is given in Attachment VI.

M.S 222 concentrations of 1, 10, and 20 mg/L did not anesthetize any of the neonates over the 48-hour exposure period (Table 7). Thirty-five percent of the organisms were anesthetized by 48 hours at 40 mg/L; none were anesthetized during the first 24 hours of exposure. All neonates (both the anesthetized and animals that were not anesthetized) survived the 48-hour exposure at 40 mg/L. As discussed in Section 2.4, viability was not evaluated via the *Daphnia* IQ Toxicity Test™ after the exposure period because <6 neonates were anesthetized at the end of the 48-hour exposure period. No neonates were anesthetized at 50 mg/L during the first 24 hours of exposure; however, all animals were anesthetized by 48 hours (Table 7). Thirty-five percent of the neonates recovered in 24 hours in clean water; however, all animals died by 48 hours.

No neonates were anesthetized at 75, 100, 125, and 150 mg/L M.S 222 during the first 4 hours of exposure (Table 7). Fifteen, 25, 65, and 75% of the neonates were anesthetized by 24 hours at 75, 100, 125, and 150 mg/L, respectively. All neonates were anesthetized and still alive after the 48-hour exposures to 75, 100, 125, and 150 mg/L. Twenty-five, 15, 20, and 20% of the neonates survived after 24 hours in clean water; no animals survived after 48 hours in clean water. All of the control animals lived during the 1-150 mg/L exposure and recovery periods and tested positively in the *Daphnia* IQ Toxicity Test™ (Table 7).

M.S 222 concentrations of 200 mg/L anesthetized all of the neonates within one hour of exposure (Table 8). All neonates were alive after 24 hours of exposure to 200 mg/L. The living animals were not mobile and were scored alive primarily because they had "twitching" antennae. Forty percent of the 1-hour exposed neonates at 200 mg/L recovered after 24 hours in clean water; 10 percent of the 2-hour exposed animals recovered after 24 hours in clean water. None of 4- and 24-hour neonates exposed to 200 mg/L recovered in 24 hours. The viability of the 1- and 2-hour exposed neonates after 24 hours recovery in clean water were tested via the *Daphnia* IQ Toxicity Test™ even though <6 neonates were alive and did not meet the minimum number for testing. Six of the eight living neonates gave a positive response after 24 hours recovery in clean water (Table 8).

Table 7. Summary of Neonates Exposed to M.S. 222 Concentrations of 1 to 150 mg/L for Periods up to 48 Hours and Number of Neonates that Recovered in Clean Water after 48 Hours

Rep	Exposure Period (No. Anesthetized/No. Alive)					Recovery in Freshwater (No. Alive)		IQ Test
	1 h	2h	4 h	24 h	48 h	24 h	48 h	(+)
					1, 10, and 20 mg/L			
A	0/10	0/10	0/10	0/10	0/10	[a]	-	-
B	0/10	0/10	0/10	0/10	0/10	[a]	-	-
					40 mg/L			
A	0/10	0/10	0/10	0/10	4/10	[a]	-	-
B	0/10	0/10	0/10	0/10	3/10	[a]	-	-
					50 mg/L			
A	0/10	0/10	0/10	0/10	10/10	3	0	-
B	0/10	0/10	0/10	0/10	10/10	4	0	-
					75 mg/L			
A	0/10	0/10	0/10	1/10	10/10	2	0	-
B	0/10	0/10	0/10	2/10	10/10	3	0	-
					100 mg/L			
A	0/10	0/10	0/10	2/10	10/10	1	0	-
B	0/10	0/10	0/10	3/10	10/10	2	0	-
					125 mg/L			
A	0/10	0/10	0/10	7/10	10/10	2	0	-
B	0/10	0/10	0/10	6/10	10/10	2	0	-
					150 mg/L			
A	0/10	0/10	0/10	7/10	10/10	2	0	-
B	0/10	0/10	0/10	8/10	10/10	2	0	-
					Controls			
A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	10/10
B	N/A	N/A	N/A	N/A	N/A	N/A	N/A	10/10

[a] Test was terminated because <50% of the neonates were anesthetized; six neonates are the minimum recommended for the *Daphnia* IQ Toxicity Test™.

Table 8. Summary of Neonates Exposed to M.S. 222 Concentrations of 200 to 800 mg/L for Periods up to 24 Hours and Number of Neonates that Recovered after 24 Hours

Exposure to Anesthesia				Recovery in Freshwater	
Exposure Period	**Rep**	**No. Anesthetized**	**No. Alive**	**No. Alive at 24 h**	**IQ Test (+)**
			200 mg/L		
1 h	A	10	10	3	3/3
	B	10	10	5	3/5
2 h	A	10	10	1	1/1
	B	10	10	1	0/1
4 h	A	10	10	0	-
	B	10	10	0	-
24 h	A	10	10	0	-
	B	10	10	0	-
			400 mg/L		
1 h	A	10	10	0	-
	B	10	10	0	-
2 h	A	10	10	0	-
	B	10	10	0	-
4 h	A	10	0	0	-
	B	10	0	0	-
24 h	A	10	0	0	-
	B	10	0	0	-
			800 mg/L		
1 h	A	10	10	0	-
	B	10	10	0	-
2 h	A	10	0	0	-
	B	10	0	0	-
4 h	A	10	0	0	-
	B	10	0	0	-
24 h	A	10	0	0	-
	B	10	0	0	-
			Controls		
N/A	N/A	N/A	N/A	N/A	10/10
N/A	N/A	N/A	N/A	N/A	10/10

All neonates were anesthetized and still alive ("twitching" antennae) in the 1-and 2-hour exposures at 400 mg/L M.S. 222; however, none of the neonates recovered after 24 hours in fresh water (Table 8). All of the neonates exposed for 4 and 24 hours at 400 mg/L were presumably anesthetized but not alive at each exposure period. All neonates were anesthetized and still alive ("twitching" antennae) in the 1-hour exposure at 800 mg/L. None of the 1-hour exposed animals recovered in 24 hours in clean water. All of the neonates in the 2-, 4-, and 24-hour exposures at 800 mg/L were presumably anesthetized but not alive at each exposure period. All of the control animals lived during the 200–800 mg/L exposure and recovery periods and tested positively in the *Daphnia* IQ Toxicity Test™ (Table 8).

The two sets of experiments with M.S. 222 show that neonates can be anesthetized; however, the animals that are anesthetized do not readily recover from the anesthesia when placed in clean water. Thus, there is no advantage in anesthetizing the neonates prior to lowering the temperatures from 20 ºC to 4-6 ºC. That is, anesthetizing neonates with M.S. 222 will not increase survival at the low temperatures. A Google Scholar™ search of the literature did not identify a single paper that addressed the use of M.S. 222 with cladoceran neonates (Google, 2006).

3.4 Production of Ephippia

The outdoor mesocosm started in mid-August and renewed with local pond water produced a limited number of males and ephippia (several dozen) by early October when the ambient temperatures began to decrease. A rapid decrease in temperature from ~ 10 ºC down to ~5 ºC overnight in mid-October caused the mesocosm to crash killing most of the adults present in the mesocosm. Thus, the evaluation was terminated.
The 1- and 4-L laboratory mesocosms (overcrowded with females) maintained under a 16h light:8h dark photoperiod did not produce any males/ephippia over an 8-week period. The 1- and 4-L laboratory mesocosms (overcrowded with females) maintained under a 24-hour continuous light photoperiod produced males and subsequent ephippia (several dozen) in approximately two weeks.

The UMD had limited success producing *D. magna* ephippia. We briefly looked at four of the six known factors listed in the Introduction that are known to initiate ephippia production. The outdoor mesocosm included overcrowding of females, reduction in temperature, and possibly the presence of bluegill kairomone. The outdoor mesocosm produced a few dozen ephippia (each contained two eggs) that appeared to be related to a decrease in ambient temperatures. Overcrowding and the possible presence of bluegill kairomone did not induce ephippia production when the ambient temperature was above 12-15 ºC. Ephippia were produced only when the ambient temperature began to drop below 12-15 ºC.

The laboratory mesocosms factors included overcrowding of females and photoperiod. No ephippia were produced when overcrowding of females and a photoperiod of 16h light:8h dark were investigated. Ephippia (few dozen) were produced in ~2 weeks when the females were overcrowded and a constant 24-hour photoperiod was used. No attempt was made to determine hatching success of the ephippia. A decision was made in consultation with representatives from USACEHR and SAIC during a conference call on November 15, 2006, not to pursue further

work on ephippia since the need for storing ephippia for extended periods of time does not appear to be necessary for the production of neonates that may ultimately may deployed in the Environmental Sentinel Biomonitor.

4.0 FUTURE RESEARCH RECOMMENDATIONS

4.1 Low Temperature Studies

The low temperature manipulation studies show that neonates (<24 hours old at the start of the temperature manipulations) can be taken from 20 ºC down to 4-6 ºC over a 24- to 26-hour period, held for 30 days at 4-6 ºC, and taken back up to 20 ºC in a 1-hour period with good survival (77-100%) and positive *Daphnia* IQ Toxicity Test™ responses (60 to 77%). With the exception of survival in neonates taken down to 6 ºC (3rd series), survival and positive response in the *Daphnia* IQ Toxicity Test™ at 60 days were poor relative to 30 days in all tests. Thus, it is recommended that neonate survival and response in the *Daphnia* IQ Toxicity Test™ be established at an intermediate exposure period (e.g., 40-45 days) between 30 and 60 days since it is necessary to establish how long the neonates can ultimately be held at 4 ºC to increase their usefulness in USACEHR's ESB.

4.2 Comparative Toxicant Testing

It is important to establish how the neonates held at low temperatures for extended periods of time respond to various toxicants. That is, do low temperature-exposed neonates respond the same as neonates held at ~20 ºC when tested via the *Daphnia* IQ Toxicity Test™? The existing *Daphnia* IQ Toxicity Test™ data base has been derived for neonates maintained and tested at room temperatures. We propose to compare existing *Daphnia* IQ Toxicity Test™ toxicant EC50s with the EC50s of neonates held at 4 ºC. USACEHR has an EC50 data base for 12 representative chemicals. Thus, we propose to use the same 12 chemicals for the comparison between the neonates held at 4 ºC with those at ~20 ºC.

Briefly, each of the 12 chemicals would be tested with neonates held at 4 ºC for 10, 20, and 30 days (and possibly 40-45 days if the above recommendation is considered). The EC50s of the low temperature neonates would be compared to EC50s established with neonates maintained and tested at ~20 ºC.

4.3 Environmental Sentinel Biomonitor

The primary goal of the current study and the proposed future research is to determine the shelf-life of the neonates at 4 ºC for potential deployment in the ESB. Since other toxicity platforms will ultimately be deployed in the ESB, it is important to establish the minimum volume that the neonates can be adequately maintained in and not affect their toxicological response. It is necessary to establish a minimum volume required for the neonates so that USACEHR can configure space and power requirements for the toxicity platforms that may ultimately be deployed in the ESB.

We propose to determine the smallest volume that neonates can be maintained in for periods of 10, 20, and 30 days (and possibly 40-45 days) and respond adequately in the *Daphnia* IQ Toxicity Test™. We performed all of the experiments in the current study in a volume of 120 mL placed in 150 mL beakers as recommended in the ASTM study protocol (ASTM, 2004). We propose to first evaluate the use of the standard 10 mL vials recommended for use in the *Daphnia* IQ Toxicity Test™. Neonates will be evaluated for periods of 10, 20, and 30 days (and possibly 40-45 days). If the neonates cannot be maintained and respond properly in the *Daphnia* IQ Toxicity Test™ in the 10 mL vials, we will increase the volume in steps of 20, 40, and 80 mL until the minimum volume is established.

We would like to discuss the following with USACEHR and SAIC. First, we have used three replicates for all exposure conditions. Does USACEHR think three replicates are satisfactory or would two be sufficient to save space and provide adequate detection of contaminants in the ESB platform? Secondly, for the sake of discussion, if three replicates are correct, should three replicates vials be used with 10 organisms/vial or should we consider one vial with 30 neonates? That is, to maximize space in the ESB, it may be more advantageous to use one vial with 30 organisms and then pipette out 6 or more neonates for each of the three replicates.

Survival and positive *Daphnia* IQ Toxicity Test™ responses should be evaluated at both 4 and 20 ºC in each test volume and compared to the data obtained in the 120 mL volume used throughout the current study. It may be advisable to run a minimum of two of USACEHR's 12 representative chemicals to confirm that volumes <120 mL do not alter the toxicological responses of the neonates. We propose that this issue be discussed with USACEHR and SAIC before any decision is made.

4.4 Supply USACEHR with 4 ºC Neonates

USACEHR has inquired about the possibility of UMD supplying neonates at 4 ºC for toxicity testing in their laboratory. UMD can provide low temperature neonates if needed.

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ATTACHMENT I

***DAPHNIA MAGNA* 21-DAY REPRODUCTION TEST DATA – FIRST SERIES (JULY 28, 2006 - AUGUST 18, 2006)**

(3 Pages)

Table I-1. Number of Broods and Neonates Produced in Each Replicate during the First Series of *Daphnia magna* 21-Day Life-Cycle Tests

Rep	Brood No. 1	Brood No. 2	Brood No. 3	Brood No. 4	Brood No. 5	Brood No. 6	Brood No. 7	No. of Broods	Total No. Young Produced
					Test 1				
A	13	17	24	26	27	31	30	7	168
B	9	24	37	18	25	36	31	7	180
C	9	29	27	19	12	33	40	7	169
D	7	19	32	21	12	22	32	7	145
E	10	19	28	19	21	24	19	7	140
F	10	24	24	20	12	26	24	7	140
G	6	24	24	21	24	23	28	7	150
H	14	27	21	22	23	19		6	126
I	8	19	34	16	19	27	12	7	135
J	9	25	28	21	24	24	21	7	152
K	13	15	31	22	16	19	17	7	133
L	9	31	26	16	13	25		6	120
					Test 2				
A	9	30	13	23	18	28	17	7	138
B	11	21	21	13	22	26	12	7	126
C	10	29	29	16	24	24	13	7	145
D	10	23	15	15	21	35	15	7	134
E	9	21	31	21	18	26	28	7	154
F	11	32	15	24	25	27	27	7	161
G	12	17	16	16	18	16	16	7	111
H	10	24	13	17	20	17	19	7	120
I	10	16	23	12	14	22	17	7	114
J	11	23	17	19	27	20	17	7	134
K	13	31	19	19	19	23	18	7	142
L	12	48	28	22	26	22		6	158

Table I-2. Water Quality during the First Series of *Daphnia magna* 21-Day Life-Cycle Tests

Parameter	Day																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
										Test 1											
Temp.[a]	20.0	20.0	20.1	20.0	20.0	20.1	20.1	20.0	20.0	20.0	20.0	20.0	20.0	20.1	20.1	20.1	20.1	20.0	20.0	20.0	20.1
D.O.[b]	8.2	8.0	8.1	8.2	8.0	8.1	7.9	8.2	8.2	7.8	8.0	8.1	8.2	8.2	8.0	7.9	8.1	8.2	8.1	8.1	8.0
pH[c]	7.76	7.71	7.80	7.82	7.70	7.83	7.77	7.82	7.69	7.76	7.83	7.87	7.77	7.81	7.72	7.80	7.85	7.72	7.79	7.86	7.82
Cond.[d]			270							270							270				270
Alkal.[e]			70							75							70				70
Hard.[f]			72							72							76				72
										Test 2											
Temp.[a]	20.0	20.0	20.1	20.0	20.0	20.1	20.1	20.0	20.0	20.0	20.0	20.0	20.0	20.1	20.1	20.1	20.1	20.0	20.0	20.0	20.1
D.O.[b]	8.3	8.1	8.0	8.0	7.9	8.0	8.0	8.1	8.2	7.7	7.9	8.1	8.0	8.2	8.1	7.8	8.0	8.1	8.1	8.2	7.9
pH[c]	7.80	7.76	7.85	7.80	7.67	7.85	7.73	7.85	7.66	7.79	7.87	7.90	7.82	7.83	7.77	7.77	7.86	7.75	7.70	7.83	7.85
Cond.[d]			270							270							270				270
Alkal.[e]			70							75							70				70
Hard.[f]			72							72							76				72

[a] Temperature units are ºC.
[b] Dissolved oxygen units are mg/L.
[c] pH units are standard units.
[d] Conductivity units are µmhos/cm.
[e] Alkalinity units are mg/L as $CaCO_3$.
[f] Hardness units are mg/L as $CaCO_3$.

ATTACHMENT II

***DAPHNIA MAGNA* 21-DAY REPRODUCTION TEST DATA – SECOND SERIES (SEPTEMBER 19, 2006 - OCTOBER 21, 2006)**

(3 Pages)

Table II-1. Number of Broods and Neonates Produced in Each Replicate during the Second Series of *Daphnia magna* 21-Day Life-Cycle Tests

Rep	Brood No. 1	Brood No. 2	Brood No. 3	Brood No. 4	Brood No. 5	Brood No. 6	Brood No. 7	No. of Broods	Total No. Young Produced
					Test 1				
A	9	9	16	17	12	13	19	7	95
B	9	9	13	12	11	15	12	7	81
C	9	14	15	15	12	12	14	7	91
D	11	16	15	16	10	13	12	7	93
E	12	14	12	13	13	14		6	78
F	10	16	12	14	13	12	14	7	91
G	9	15	13	13	13	15		6	78
H	14	14	14	15	18	15		6	90
I	12	14	9	9	17	17	15	7	93
J	7	13	14	9	14	17	14	7	88
K	9	15	12	12	11	17	12	7	88
L	10	17	12	13	15	18		6	85
					Test 2				
A	11	10	11	17	17	14	12	7	92
B	7	17	15	16	8	17	14	7	94
C	11	16	11	14	12	16		6	80
D	13	15	14	16	14	13	11	7	96
E	9	15	13	16	13	15		6	81
F	8	12	15	15	14	15		6	79
G	16	15	16	10	18	17		6	92
H	8	10	16	15	17	17	14	7	97
I	10	8	16	12	13	16	15	7	90
J	11	13	11	13	11	16	14	7	89
K	7	16	17	16	12	14	16	7	98
L	9	15	15	13	13	16	13	7	94

Table II-2. Water Quality during the Second Series of *Daphnia magna* 21-Day Life-Cycle Tests

Parameter	Day																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Test 1																					
Temp.[a]	20.0	20.1	20.1	20.0	20.0	20.1	20.1	20.0	20.0	20.0	20.0	20.0	20.1	20.1	20.1	20.1	20.1	20.0	20.0	20.0	20.1
D.O.[b]	8.0	7.9	7.9	8.0	8.1	7.9	8.1	8.0	7.8	8.0	8.1	7.9	7.9	8.1	8.0	7.8	7.7	8.0	7.8	7.7	7.9
pH[c]	7.15	7.71	7.75	7.70	7.65	7.70	7.63	7.74	7.71	7.75	7.77	7.80	7.69	7.70	7.72	7.69	7.74	7.80	7.70	7.69	7.79
Cond.[d]	270							270							280						270
Alkal.[e]	70							70							70						70
Hard.[f]	76							72							72						72
Test 2																					
Temp.[a]	20.0	20.1	20.1	20.0	20.0	20.1	20.1	20.0	20.0	20.0	20.0	20.0	20.1	20.1	20.1	20.1	20.1	20.0	20.0	20.0	20.1
D.O.[b]	8.1	7.7	7.9	8.0	8.0	7.7	7.9	8.1	8.0	7.9	8.0	8.1	7.8	8.0	7.7	7.8	7.9	8.1	7.7	7.8	7.8
pH[c]	7.71	7.75	7.81	7.70	7.67	7.77	7.72	7.82	7.84	7.79	7.82	7.79	7.66	7.69	7.66	7.73	7.78	7.85	7.76	7.73	7.82
Cond.[d]	270							210							280						270
Alkal.[e]	70							70							70						70
Hard.[f]	76							72							72						72

[a] Temperature units are ºC.
[b] Dissolved oxygen units are mg/L.
[c] pH units are standard units.
[d] Conductivity units are µmhos/cm.
[e] Alkalinity units are mg/L as $CaCO_3$.
[f] Hardness units are mg/L as $CaCO_3$.

ATTACHMENT III

***DAPHNIA MAGNA* 21-DAY REPRODUCTION TEST DATA – THIRD SERIES (OCTOBER 16, 2006 - NOVEMBER 6, 2006)**

(3 Pages)

Table III-1. Number of Broods and Neonates Produced in Each Replicate During the Third Series of *Daphnia magna* 21-Day Life-Cycle Tests

Rep	Brood No. 1	Brood No. 2	Brood No. 3	Brood No. 4	Brood No. 5	Brood No. 6	Brood No. 7	No. of Broods	Total No. Young Produced
					Test 1				
A	9	12	11	25	12	29		6	98
B	15	12	13	30	11	14		6	95
C	9	13	10	18	10	15		6	75
D	8	13	10	14	16	12	18	7	91
E	8	9	11	22	10	12		6	72
F	17	17	10	16	11	14		6	85
G	12	12	15	13	15	15		6	82
H	16	16	10	19	14	13		6	88
I	13	7	16	26	11	14		6	87
J	7	10	16	17	10	16		6	76
K	8	10	13	28	11	12		6	82
L	12	10	10	12	14	13		6	71
					Test 2				
A	9	8	17	17	21	15	14	7	101
B	10	17	10	10	22	16		6	85
C	9	12	14	19	10	19		6	83
D	13	10	14	14	11	13		6	75
E	9	10	10	20	11	19		6	79
F	8	9	12	12	11	13	12	7	77
G	8	12	14	17	11	22		6	84
H	8	19	16	15	12	14		6	84
I	14	9	10	12	16	13		6	74
J	11	13	17	10	12	11	14	7	88
K	10	9	15	20	13	18		6	85
L	9	9	10	14	10	19		6	71

Table III-2. Water Quality during the Third Series of *Daphnia magna* 21-Day Life-Cycle Tests

Parameter	Day																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Test 1																					
Temp.[a]	20.0	20.0	20.1	20.0	20.0	20.1	20.1	20.0	20.0	20.0	20.0	20.0	20.0	20.1	20.1	20.1	20.1	20.0	20.0	20.0	20.0
D.O.[b]	7.9	8.1	7.8	7.8	8.0	8.1	8.1	8.0	7.8	7.7	7.9	7.7	7.8	7.9	7.8	8.0	7.9	7.8	7.9	7.9	7.7
pH[c]	7.70	7.76	7.82	7.72	7.75	7.81	7.86	7.73	7.70	7.73	7.80	7.76	7.83	7.77	7.79	7.84	7.80	7.86	7.77	7.82	7.88
Cond.[d]	270							280							270						280
Alkal.[e]	70							70							70						75
Hard.[f]	72							68							72						76
Test 2																					
Temp.[a]	20.0	20.0	20.1	20.0	20.0	20.1	20.1	20.0	20.0	20.0	20.0	20.0	20.0	20.1	20.1	20.1	20.1	20.0	20.0	20.0	20.0
D.O.[b]	7.8	8.0	8.0	7.9	8.0	8.0	8.1	8.0	7.9	7.8	7.8	7.8	7.9	8.0	7.9	7.9	7.9	8.0	8.0	7.9	7.8
pH[c]	7.75	7.75	7.86	7.77	7.80	7.86	7.77	7.73	7.70	7.76	7.83	7.73	7.82	7.83	7.76	7.82	7.81	7.83	7.73	7.80	7.85
Cond.[d]	270							280							270						280
Alkal.[e]	70							70							70						75
Hard.[f]	72							68							72						76

[a] Temperature units are ºC.
[b] Dissolved oxygen units are mg/L.
[c] pH units are standard units.
[d] Conductivity units are µmhos/cm.
[e] Alkalinity units are mg/L as $CaCO_3$.
[f] Hardness units are mg/L as $CaCO_3$.

ATTACHMENT IV

***DAPHNIA MAGNA* 21-DAY REPRODUCTION TEST DATA – FOURTH SERIES (NOVEMBER 6, 2006 - NOVEMBER 27, 2006)**

(3 Pages)

Table IV-1. Number of Broods and Neonates Produced in Each Replicate During the Fourth Series of *Daphnia magna* 21-Day Life-Cycle Tests

Rep	Brood No. 1	Brood No. 2	Brood No. 3	Brood No. 4	Brood No. 5	Brood No. 6	Brood No. 7	No. of Broods	Total No. Young Produced
					Test 1				
A	11	8	18	28	16	12		6	93
B	10	18	14	13	19	20		6	94
C	13	16	12	28	25	13		6	107
D	7	10	13	25	25	19		6	99
E	11	23	30	24	15	12		6	115
F	11	12	7	13	19	20	12	7	94
G	10	12	22	28	14	18		6	104
H	7	20	31	23	17	13		6	111
I	12	17	33	16	19	14		6	111
J	10	19	11	26	27	13		6	106
K	11	19	17	17	24	18		6	106
L	10	15	23	22	17	14		6	101
					Test 2				
A	10	11	14	18	17	19		6	89
B	9	12	15	16	15	14	22	7	103
C	8	10	14	30	14	22	20	7	118
D	11	24	11	32	14	31		6	123
E	9	14	24	12	22	14		6	95
F	12	26	11	36	20	12		6	117
G	8	12	24	13	21	18		6	96
H	13	21	11	34	12	15		6	106
I	11	10	13	30	13	20	22	7	119
J	10	22	11	27	14	13		6	97
K	12	14	24	13	19	23		6	105
L	15	27	10	30	11	14		6	107

Table IV-2. Water Quality during the Fourth Series of *Daphnia magna* 21-Day Life-Cycle Tests

Parameter	Day																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Test 1																					
Temp.[a]	20.0	20.0	20.0	20.0	20.0	20.1	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.1	20.1	20.1	20.0	20.0	20.0	20.0	20.0
D.O.[b]	7.9	7.8	7.8	7.9	7.7	7.7	7.9	7.6	7.9	8.0	7.8	7.9	8.0	7.7	7.6	7.5	7.8	7.7	7.9	7.8	7.9
pH[c]	7.70	7.62	7.71	7.70	7.65	7.60	7.69	7.71	7.73	7.65	7.63	7.74	7.70	7.64	7.67	7.59	7.66	7.61	7.70	7.63	7.67
Cond.[d]	270							270							280						280
Alkal.[e]	75							75							75						75
Hard.[f]	72							76							76						72
Test 2																					
Temp.[a]	20.0	20.0	20.0	20.0	20.0	20.1	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.1	20.1	20.1	20.0	20.0	20.0	20.0	20.0
D.O.[b]	7.9	7.7	7.7	7.5	7.9	7.5	7.6	7.7	7.9	7.9	7.6	7.5	7.6	7.8	7.7	7.6	7.9	7.7	7.7	7.7	7.9
pH[c]	7.67	7.62	7.71	7.67	7.61	7.60	7.63	7.65	7.70	7.66	7.69	7.64	7.67	7.61	7.70	7.73	7.62	7.65	7.62	7.73	7.66
Cond.[d]	280							280							280						280
Alkal.[e]	75							75							75						75
Hard.[f]	72							76							72						72

[a] Temperature units are ºC.
[b] Dissolved oxygen units are mg/L.
[c] pH units are standard units.
[d] Conductivity units are µmhos/cm.
[e] Alkalinity units are mg/L as $CaCO_3$.
[f] Hardness units are mg/L as $CaCO_3$.

ATTACHMENT V

WATER QUALITY DURING THE *DAPHNIA MAGNA* TEMPERATURE EXPOSURES

(3 Pages)

Table V-1. Water Quality during the *Daphnia magna* Temperature Exposures

Exposure Period	pH (Standard Units)			Dissolved Oxygen (mg/L)			Conductivity (µmhos/cm)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
	Start of Expos. 20 ºC	End of Expos. 4-6 ºC	End of Expos. 20 ºC	Start of Expos. 20 ºC	End of Expos. 4-6 ºC	End of Expos. 20 ºC	Start of Expos. 20 ºC	Start of Expos. 20 ºC	Start of Expos. 20 ºC
Series 1 (20 to 4 ºC in 1 Hour; return to 20 º in 1 Hour)									
Day 10	7.77	7.68	7.69	8.0	7.8	7.7	270	70	76
Day 20	7.77	7.77	7.78	8.0	7.7	7.6	270	70	76
Day 30	7.77	7.79	7.80	8.0	7.5	7.3	270	70	76
Series 2 (20 to 4 ºC in 6 Hours; return to 20 º in 6 Hours)									
Day 10	7.87	7.81	7.82	8.1	7.8	7.7	270	70	72
Day 20	7.87	7.79	7.79	8.1	7.6	7.5	270	70	72
Day 30	7.87	7.85	7.84	8.1	7.4	7.0	270	70	72
Series 3 (20 to 6 ºC in 25 Hours; return to 20 º in 6 Hours)									
Day 10	7.65	7.61	7.59	7.9	7.5	7.0	270	70	72
Day 20	7.65	7.61	7.57	7.9	7.5	7.0	270	70	72
Day 30	7.65	7.45	7.42	7.9	7.4	7.1	270	70	72
Day 60	7.65	7.46	7.37	7.9	7.3	6.8	270	70	72
Series 4 (20 to 4 ºC in 26 Hours; return to 20 º in 1 Hour)									
Day 10	7.79	7.77	7.75	8.0	7.8	7.7	280	75	76
Day 20	7.79	7.76	7.74	8.0	7.6	7.3	280	75	76
Day 30	7.79	7.65	7.61	8.0	7.5	7.0	280	75	76
Day 60	7.79	7.36	7.29	8.0	7.3	6.3	280	75	76

Table V-1. Water Quality during the *Daphnia magna* Temperature Exposures (Continued)

Exposure Period	pH (Standard Units)			Dissolved Oxygen (mg/L)			Conductivity (µmhos/cm)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
	Start of Expos. 20 ºC	End of Expos. 4 ºC	End of Expos. 20 ºC	Start of Expos. 20 ºC	End of Expos. 4 ºC	End of Expos. 20 ºC	Start of Expos. 20 ºC	Start of Expos. 20 ºC	Start of Expos. 20 ºC
Series 5 (20 to 4 ºC in 24 Hours; return to 20 º in 1 Hour) - ~24-Hour Old Neonates									
Day 10	7.70	7.43	7.39	7.9	7.4	6.9	280	75	72
Day 20	7.70	7.41	7.33	7.9	7.5	6.8	280	75	72
Day 30	7.70	7.37	7.29	7.9	7.5	6.9	280	75	72
Day 60	7.70	7.28	7.16	7.9	7.2	6.6	280	75	72
Series 5 (20 to 4 ºC in 24 Hours; return to 20 º in 1 Hour) - ~48-Hour Old Neonates									
Day 10	7.72	7.49	7.47	8.2	8.1	7.9	280	75	72
Day 20	7.72	7.39	7.32	8.2	7.9	7.3	280	75	72
Day 30	7.72	7.34	7.26	8.2	7.2	6.7	280	75	72
Day 60	7.72	7.27	7.20	8.2	7.0	6.3	280	75	72
Series 5 (20 to 4 ºC in 24 Hours; return to 20 º in 1 Hour) - ~96-Hour Old Neonates									
Day 10	7.69	7.31	7.17	8.0	7.5	7.0	280	75	76
Day 20	7.69	7.36	7.28	8.0	7.6	7.2	280	75	76
Day 30	7.69	7.33	7.26	8.0	7.3	6.8	280	75	76
Day 60	7.69	7.29	7.13	8.0	7.1	6.5	280	75	76

ATTACHMENT VI

WATER QUALITY DURING THE *DAPHNIA MAGNA* ANESTHETIC (M.S. 222) EXPOSURES

(2 Pages)

Table VI-1. Water Quality during the *Daphnia magna* M.S. 222 Exposures

Conc. (mg/L)	Ph (Stand. Units)		D.O. (mg/L)		Conductivity (µmhos/cm)		Alkalinity (mg/L as $CaCO_3$)		Hardness (mg/L as $CaCO_3$)	
	Start of Expos.	Start of Recov.	Start of Expos.	Start of Recov.	Start of Expos.	Start of Recov.	Start of Expos.	Start of Recov.	Start of Expos.	Start of Recov.
1 - 150 mg/L Series										
1	7.82	N/A	8.3	N/A	280	N/A	75	N/A	72	N/A
10	7.80	N/A	8.3	N/A	280	N/A	75	N/A	72	N/A
20	7.76	N/A	8.3	N/A	280	N/A	75	N/A	72	N/A
40	7.76	N/A	8.3	N/A	280	N/A	75	N/A	72	N/A
50	7.73	7.82	8.2	8.4	270	280	70	70	76	76
75	7.71	7.82	8.3	8.3	270	280	70	70	76	76
100	7.68	7.82	8.2	8.3	270	280	70	70	76	76
125	7.76	7.81	8.2	8.3	270	280	70	70	76	76
150	7.62	7.81	8.2	8.2	270	280	70	70	76	76
Control	7.75	7.82	8.3	8.3	280	280	75	70	72	76
200 - 800 mg/L Series										
200	7.53	7.82	8.1	8.2	280	280	75	70	72	76
400	7.46	7.81	8.1	8.2	280	280	75	70	72	76
800	7.33	7.81	8.0	8.2	280	280	75	70	72	76
Control	7.83	7.81	8.3	8.4	280	280	75	70	72	76